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Comparison of Primer Sets for Detection of Fecal and Ocular Adenovirus Infection Using the Polymerase Chain Reaction

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Adenoviruses of subgenus F (types 40 and 41) cause infantile gastroenteritis and adenoviruses principally of types 1–7 are found in feces during respiratory or generalized infections. Adenoviruses (mostly types 3, 4, 8, 19, or 37) are also linked with follicular or epidemic conjunctivitis. The diagnostic efficiency of the polymerase chain reaction (PCR) for adenoviruses was assessed using genus-reactive primers H1 and H2 or JCH1 and JCH2 or subgenus F-specific primers F1a and F2a. With diarrheal stool specimens containing subgenus F adenoviruses, F1a/F2a PCR achieved at least as high a positivity rate (75/76 [99%]) as electron microscopy (72/76 [95%]) and was more sensitive than polyclonal antibody-based immune electron microscopy (IEM) for subgenus identification (75/76 [99%] vs. 66/76 [87%], $P = 0.008$). Twenty-three subgenus F strains untypeable by monoclonal antibody-based IEM were typed as 40 (n = 4) or 41 (n = 19) by *Hha* I digestion of the PCR product. The genus-reactive primer pairs provided DNA amplification assays of generally equal efficiency on conjunctival swab specimens though possible nucleic acid degradation in DNA extracts during storage could have meant that JCH1/JCH2 PCR was truly the more sensitive. The use of either genus-reactive primer set on fecal specimens cannot be recommended because, although the positivity rates with subgenus F PCR positive specimens were high (70/75 [93%] for H1 and H2, 14/15 [93%] for JCH1 and JCH2), the detection rates were disappointing with similar specimens yielding non-subgenus F adenoviruses.

KEY WORDS: polymerase chain reaction, primer sets, fecal adenovirus infection, ocular adenovirus infection, rapid diagnosis

INTRODUCTION

Adenoviruses are associated with follicular conjunctivitis (types 3 and 7 [subgenus B] and 4 [subgenus E]), epidemic keratoconjunctivitis (EKC) (types 8, 19, and 37 [subgenus D]), infantile gastroenteritis (types 40 and 41 [subgenus F]), and respiratory tract and generalized infections (types 1, 2, 5, and 6 [subgenus C] and types 3 and 7 [subgenus B] [Horwitz, 1990]). Rapid detection of adenoviruses in conjunctival swabs aids the early clinical management of EKC outbreaks [Ankers et al., 1993]. Adenovirus subgenus F strains are second only to rotavirus in the causation of infantile diarrhea [Kidd et al., 1986; Bates et al., 1993]. Adenovirus in fecal specimens might also be causing respiratory disease in immunocompetent or disseminated infections in immunocompromised patients [Horwitz, 1990; Johnson et al., 1990; Morris et al., 1993].

Detection of adenoviruses in conjunctival swabs relies on virus isolation in cell culture or antigen detection [Sharp and Bailey, 1988; Killough et al., 1990]. These tests have the respective disadvantages of tardiness or low sensitivity and specificity. Electron microscopy (EM), immune EM (IEM), or enzyme-linked immunosorbent assay (ELISA) [Wood and Bailey, 1987; Wood et al., 1989a,b] are employed to detect adenovirus types 40 and 41 in fecal samples, though only the latter two differentiate between these serotypes and others less likely to cause diarrhea. Identification and typing of subgenus F adenoviruses by IEM give both false negative and inaccurate results in comparison with the reference technique of restriction endonuclease analysis (REA) of adenovirus genomic DNA using *Sma* I [Wood and Bailey, 1987; Wood et al., 1989a].

The polymerase chain reaction (PCR) is a sensitive,

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specific, and rapid method applicable to the amplification of adenovirus DNA. In this study, two sets of genus-reactive primers, H1 and H2 [Allard et al., 1990] or JCH1 and JCH2 [Hierholzer et al., 1993] were compared on conjunctival swabs, and these two and a third set of subgenus F-specific primers F1 and F2 [Rousell et al., 1993] on fecal samples. The H1/H2 PCR is more sensitive than antigen detection for ocular adenoviruses, but an alternative genus-reactive assay might avoid false negative results, perhaps paricularly with subgenus B strains [Morris et al., 1995]. Fecal samples could be screened for adenoviruses by genus-reactive PCR, subgenus F-specific PCR, or a combination of the two. In addition, REA of the subgenus F PCR product was evaluated as an alternative to IEM or *Sma* I REA for typing of subgenus F adenoviruses detected in fecal specimens.

MATERIALS AND METHODS

Clinical Samples

Cotton-tipped conjunctival swabs from patients with eye disease were collected in virus transport medium [Killough et al., 1990]. Fecal specimens from patients with gastroenteritis, respiratory, or generalized infections were emulsified in feces transport medium [Wood and Bailey, 1987].

Virus isolation was attempted on each eye swab transport medium by immediate inoculation of monolayer cultures of human embryo lung fibroblast, Vero, and Hep-2 cells [Killough et al., 1990]. These cultures were discarded after 1 week as negative if the genus-reactive adenovirus PCR using primers H1 and H2 gave a negative result. This PCR proved overall as sensitive as virus culture for adenoviruses, and culture was only retained with PCR negative samples for detection of herpes simplex virus [Morris et al., 1995]. If the H1/H2 PCR was positive, the cell cultures were maintained for up to 4 weeks in an attempt to identify the adenovirus and type it by neutralization [Killough et al., 1990]. Adenovirus particles were sought in diarrheal stool specimens by EM and were identified as subgenus F strains by IEM using a polyclonal rabbit antiserum [Wood and Bailey, 1987]. Subgenus F adenoviruses were serotyped by IEM with monoclonal type-specific antibodies [Wood et al., 1989a] and/or by *Sma* I REA [Wood and Bailey, 1987]. Stool specimens from patients with symptoms additional to diarrhea and vomiting (e.g., cough, coryza, pneumonia, encephalitis) were also inoculated immediately onto the aforementioned cell cultures which were maintained for 3 weeks prior to discard as negative [Wood and Bailey, 1987].

Residual fecal samples or eye swabs, after storage at −30°C for up to 10 years or 4°C for up to 7 days, respectively, were prepared for PCR testing by mixing in equal volumes (40 μl) with lysis buffer (20 mM Tris hydrochloric acid [Tris-HCl] pH 8.3, 2 mM ethylenediaminetetraacetic acid [EDTA], 1% Triton X-100, 0.002 g/l sodium dodecyl sulfate [SDS], 500 μg/ml proteinase K), heating at 56°C for 2 hours, and then boiling for 10 minutes [Morris et al., 1995]. Alternative preparation protocols evaluated on a limited number of fecal emulsions were boiled for 10 minutes with or without subsequent 1 : 10 dilution in sterile distilled water (SDW) and proteinase K/SDS digestion followed by phenol-chloroform extraction [Kimpton et al., 1988]. The DNA extracts were tested within a few days by PCR using primers F1 and F2, F1a and F2a, or H1 and H2, but using primers JCH1 and JCH2 only after storage at −30°C for 1–2 years.

Primers

Three sets of primers were evaluated (Pharmacia Biotech, Milton Keynes, UK). Two genus-reactive sets derived from the shared DNA sequences of adenovirus types 2 and 5 [Kinloch et al., 1984] were used for conjunctival swabs and these and a third subgenus F-specific pair were used for fecal samples. The first set was H1-5′ GCCGCAGTGGTCTTACATGCACATC 3′ (position 18858–18883, 25 Mer) and H2- 5′ CAGCACGCCGCGGATGTCAAAGT 3′ (position 19158–19135, 23 Mer), yielding a product of 300 base pairs [Allard et al., 1990]. The second set was JCH1-5′ GCCGAGAAGGGCGTGCGCAGGTA 3′ (position 21725–21703, 23 Mer) and JCH2-5′ TACGCCAACTCCGCCCACGCGCT 3′ (position 21565–21587, 23 Mer), with a product of 161 base pairs [Hierholzer et al., 1993]. The published subgenus F-specific set had the sequence F1- 5′ GTCACGTTCAGAGTTGCTGCATAT 3′ (position 3077–3053, 24 Mer) and F2- 5′ GTCCCAGTGTTAAGCATAATATG 3′ (position 2689–2711, 23 Mer) [Rousell et al., 1993], but when used the sensitivity was poor. Mistakes in the sequences were noted at the underlined positions (3057 in F1, 2689 and 2691 in F2) where the primers differed from the published shared sequences of adenovirus types 40 and 41 [van Loon et al., 1987]. The primers were then corrected to F1a- 5′ GTCACGTTCAGAGTTGCTGGATAT 3′ (position 3077–3053, 24 (Mer) and F2a- 5′ CTGCCAGTGTTAAGCATAATATG 3′ (position 2689–2711, 23 Mer), so that both primers comprised sequences almost entirely shared between prototype adenovirus type 40 and 41 strains except in F2a at positions 2699 (40 = A, 41 = T) and 2708 (40 = C, 41 = T). These primers yielded a product of 388 base pairs.

PCR Amplification

The reaction mixture for primer pair JCH1 and JCH2 comprised 10 mM Tris-HC1 (pH 8.3), 50 mM potassium chloride, 2 mM magnesium chloride ($MgCl_2$), 200 μM deoxynucleoside triphosphates (dNTPs), 0.2 μM primers, 1.25 units AmpliTaq DNA polymerase (Perkin-Elmer-Cetus, Warrington, England) (total volume 45 μl), 5 μl DNA extract or SDW (included in every assay as a negative control), and a mineral oil overlay as previously optimized for primers H1 and H2 [Turner et al., 1993; Morris et al., 1995]. With primers H1 and H2, F1 and F2, and F1a and F2a, the reaction constituents comprise 67 mM Tris-HC1 (pH 8.4), 16 mM ammonium sulfate, 2 mM $MgC1_2$, 0.17% bovine serum albumin, 200 μM dNTPs, 0.2 μM primers, 1 unit AmpliTaq DNA polymerase (total volume 45 μl), and 5 μl DNA extract or SDW.

The latter PCR mixture was in routine use in our diagnostic laboratory, being based on that described by Kimpton et al. [1991] for human cytomegalovirus DNA. The two sets of reagents gave similar results with adenovirus primers H1 and H2. The anticontamination measures used for the three PCRs were those described previously for the H1/H2 DNA amplification assay [Turner et al., 1993; Morris et al., 1995].

All three PCRs relied on 94°C and 72°C for 1 minute as denaturation and extension steps following initial denaturation for 7 minutes, with 40 cycles and annealing at 40°C for 1 minute (primers H1 and H2) or 30 cycles and annealing at 55°C for a similar time (primers JCH1 and JCH2). Thirty and later 50 cycles with an annealing temperature of 40°C for 1 minute were used for the subgenus F-specific primers.

Electrophoresis

After adding 2 μl loading buffer (0.25% bromophenol blue, 40% sucrose) to 10 μl amplified DNA the products were electrophoresed in 1.8% agarose (100 V for 1 hour, all PCRs using primers H1 and H2, F1 or F2, or F1a and F2a), 3% Nu-Sieve (75 V for 1 hour), or 6% polyacrylamide gels (200 V for 20 min). All gels included a 1 kb ladder (Gibco-BRL, Paisley, UK) to confirm the size of the DNA products. Gels were stained in 1 × TAE (0.04 M Tris acetate, 0.001 M EDTA) containing 5 μg/ml ethidium bromide and examined under ultraviolet (UV) transilluminator light for the presence of DNA bands.

PCR Typing of Subgenus F Adenoviruses

The 388 base pair F1a/F2a PCR product was identified in the published DNA sequences of adenovirus types 40 and 41 [van Loon et al., 1987]. Analysis on PC/GENE (Intelligenetics, Oxford, UK) identified 1 or 2 *Hha* I restriction endonuclease cut-sites in these 2 sequences, producing fragments of 151 and 237 (type 40) or 60, 52, and 276 base pairs (type 41). For PCR typing, the F1a/F2a reaction product (20 μl volume) was therefore cleaved at 37°C overnight using 5 units *Hha* I (Pharmacia). The resultant fragments were separated electrophoretically in a 15% polyacrylamide gel at 180 V for 45 minutes. Molecular weight markers (pBR322 *Hae* III digest; Sigma, Poole, UK) were used to confirm the size of the DNA fragments.

Control Samples

Purified genomic adenovirus type 2 DNA (Gibco-BRL) was used as target in the optimization of the JCH1/JCH2 PCR and as positive control (4,000 copies) in each assay run with these primers. Lysis buffer-extracted adenovirus type 2-infected cells (10^5 per reaction mixture) provided the positive control for the H1/H2 PCR. Positive controls for the F1/F2 and F1a/F2a PCRs comprised two stool samples containing adenovirus type 40 and two containing adenovirus type 41 identified by IEM [Wood et al., 1989a] and *Sma* I REA [Wood and Bailey, 1987]. A stool sample negative for adenoviruses by EM and virus culture provided a negative control.

Statistical Analysis

The results of the adenovirus PCRs were analyzed using the two-tailed Fisher's exact test.

RESULTS

Optimization and Sensitivity of PCR Using Primers JCH1 and JCH2

The optimal $MgCl_2$ concentration of 2 mM, determined by using different $MgCl_2$ concentrations with 0.2 μM primers, gave a detection limit of less than 400 genome copies.

Optimization of Subgenus F-Specific PCR

Using primers F1 and F2 and 30 or even 50 thermal cycles, the PCR gave positive results with only 7 of 13 IEM positive fecal specimens. The results were identical irrespective of whether specimens were prepared by heat alone, heat and dilution, phenol-chloroform extraction, or lysis buffer extraction. Lysis buffer treatment was therefore used in all subsequent experiments because of its simplicity and proven effectiveness with eye swabs.

Following identification of the errors in the primer set F1 and F2 (see Materials and Methods), switching to corrected primers F1a and F2a gave more positive PCR results (n = 12) with the aforementioned 13 fecal samples. Eight strains were identified as types 40 (n = 6) or 41 (n = 2) by IEM, but all 13 were so classified by *Sma* I REA (6 as type 40 and 7 as type 41), including 1 typed as 40 by IEM but 41 by REA. Two specimens gave weakly positive DNA bands after 30 thermal cycles but much more intense bands after 50 cycles. All subsequent subgenus F-specific PCRs therefore relied on primers F1a and F2a with 50 thermal cycles.

Lysis buffer extracts of 10^5 cells infected with adenoviruses representative of subgenera A (type 31), B (type 7), C (type 2), D (type 10), and E (type 4) all gave negative results in DNA amplification assays with primers F1a and F2a when positive results were obtained using the H1 and H2 primers on the same material.

Retrospective Comparison of PCRs on Eye Swabs

Among an initial set of 20 culture positive swabs selected as having a low reactivity rate (7, 35%) with primers H1 and H2, 15 (75%) were positive with primers JCH1 and JCH2 when the products were electrophoresed on 1.8% agarose gels, and 1 extra sample (giving a total of 16 [80%], $P = 0.02$ for comparison with H1/H2 PCR) was positive on 6% polyacrylamide gels (Table I). Electrophoresis on a 3% Nu-Sieve gel revealed the same extra positive result (Table I). Henceforth, all JCH1/JCH2 PCR products were analyzed using polyacrylamide gels, because visualization of the small 161 base pair product was easier than on agarose gels and Nu-Sieve gels were unacceptably expensive. Adenovirus PCR using primers JCH1 and JCH2 and polyacrylamide gel electrophoresis (PAGE) gave more positive results than H1/H2 PCR for 13 subgenus B (type 3 or 7) strains

TABLE I. Results of Retrospective Study on 20 Eye Swabs Having Low Adenovirus PCR Positivity Rate With Primers H1 and H2*

Virus isolation	PCR using primers H1 and H2	PCR using primers JCH1 and JCH2			No. of samples
		Agarose	Nu-Sieve	PAGE	
Ad 2	–	–	–	–	2
Ad 3	+	+	NT	+	5
	–	+	+	+	4
	–	–	–	–	2
Ad 4	+	+	NT	+	2
	–	–	+	+	1
Ad 7	–	+	+	+	2
Ad 10	–	+	+	+	1
Ad (not typed)	–	+	+	+	1

*Ad 2, 3, 4, 7, 10, (not typed): adenovirus types 2, 3, 4, 7, 10, or untyped. – = negative; + = positive; NT = not tested. Products electrophoresed on agarose, Nu-Sieve, or polyacrylamide gels (PAGE) where shown.

(11 [85%] vs. 5 [38%], $P = 0.04$) and possibly for 7 other adenoviruses (5[71%] vs 2[29%], $P > 0.1$).

With a second set of 21 culture positive samples selected as having a high reactivity rate (19, 90%) with primers H1 and H2 only 9 positive results (43%, $P = 0.002$) were obtained using primers JCH1 and JCH2. The first primer pair gave only 2 more positive results than the second set with 9 subgenus B (type 3 or 7) strains (7 [78%] vs. 5 [56%], $P > 0.1$), but a large difference was observed with 12 other adenoviruses (all but 2 of subgenus C) (12 [100%] vs. 4 [33%], $P = 0.01$).

With all 41 conjunctival swab samples, primers H1 and H2 gave only 4 less positive results than primers JCH1 and JCH2 with 22 specimens yielding subgenus B strains (12 [55%] vs. 16 [73%], $P > 0.1$). A reversed but also non-significant difference in sensitivity between the 2 primer pairs was seen with 19 other adenovirus strains (14 [74%] vs. 9 [47%], $P = 0.1$).

As the DNA extracts had been stored between testing using the H1/H2 and JCH1/JCH2 PCRs, we reexamined 4 of the second batch of DNA extracts initially positive with the former primer set where the residual volume was adequate for retesting. Only two then gave adenovirus bands suggesting degradation of viral DNA in the other two extracts during storage.

Prospective Comparison of PCRs on Eye Swabs

Over a 3 month period, 157 eye swabs were tested prospectively by both genus-reactive PCRs within 1 week of receipt and also virus isolation. Fifteen gave positive results. Seven were positive by virus isolation (adenovirus type 3 = 5, untyped strains = 2), and PCRs on these samples gave 5 or 7 positive results with primers H1 and H2 or JCH1 and JCH2 (sensitivities 71% vs. 100%), $P > 0.1$). Of eight isolation negative PCR positive swabs, three, three, or two were positive using both sets of primers, only JCH1 and JCH2, or only H1 and H2, respectively. All specimens giving PCR positive isolation negative results were obtained from patients with follicular conjunctivitis. Twelve swabs were herpes simplex virus (HSV) positive by virus culture and negative in both PCRs. The remaining 130 were negative in all tests.

Retrospective Study of PCRs on Fecal Samples

All 127 fecal specimens were tested by PCR using 2 sets of primers (H1 and H2, F1a and F2a), and 34 were also examined with primers JCH1 and JCH2. The samples were selected for testing and categorized into four groups on the basis of the results of virus isolation, EM, IEM, and REA using *Sma* I. The first group comprised 66 specimens from patients with diarrhea. All yielded adenovirus particles by EM and these strains were all identified as adenovirus subgenus F strains by IEM. Forty-nine of the adenoviruses were typed by REA using *Sma* I digestion, revealing 14 type 40 and 35 type 41 strains. Adenovirus typing by IEM gave results identical to *Sma* I REA with 17 adenoviruses (type 40 = 7, type 41 = 10). Two strains were typed as 40 by REA but 41 by IEM, and one strain was typed as 41 by REA but 40 by IEM. The PCRs using primers F1a and F2a, H1 and H2, or JCH1 and JCH2 gave positive results with virtually all specimens examined from this group (sensitivities 65/66 [98%], 65/66 [98%], and 11/11 [100%], respectively).

The second group of 19 fecal specimens also from patients with diarrhea revealed adenovirus particles by EM but these viruses could not be identified as subgenus F (type 40 and 41) strains by IEM. Both initial and repeat DNA extracts on 6 specimens contained subgenus F adenovirus DNA detected by PCR using primers F1a and F2a, and, on these fecal extracts, the sensitivities with primers H1 and H2 or JCH1 and JCH2 were identical (4/6 [67%] and 2/3 [67%], respectively) (Table II). With the remaining specimens negative by subgenus F PCR the positivity rates in the 2 other PCRs were not significantly different from each other (in order 8/13 [62%] vs. 1/4 [25%], $P > 0.1$), but the first assay was less sensitive than EM ($P = 0.04$). The negative adenovirus subgenus F PCR results on some EM positive, IEM negative samples did not reflect a particularly long storage time before DNA testing (data not shown).

The third group of 23 stool specimens from patients with diarrhea and/or other symptoms was negative or not tested by EM, but yielded adenoviruses of various

TABLE II. Results of Retrospective Study on EM Positive, IEM Negative Fecal Samples*

PCR using primers F1a and F2a	PCR using primers H1 and H2	PCR using primers JCH1 and JCH2	No. of samples
+[a]	+	+	2
+[a]	+	NT	2
+[a]	−	−	1
+[a]	−	NT	1
−	+	+	1
−	+	NT	7
−	−	−	3
−	−	NT	2

*+ = positive; − = negative; NT = not tested.
[a]Identical result on two DNA extracts.

types in cell culture (Table III). Three specimens revealed either adenovirus type 1 or 3 by virus isolation, and subgenus F adenovirus DNA by PCR in both initial and repeat extracts. After DNA amplification, more positive results were produced with the remaining samples using primers H1 and H2 than with primers JCH1 and JCH2 (10/20 [50%] vs. 1/11 [9.1%], $P = 0.05$), with both primer pairs providing a less sensitive test than virus culture ($P < 0.001$ and $P = 0.001$). Four of seven specimens positive with the first but not the second set of genus-reactive primers yielded subgenus C (type 1, 2, 5, or 6) strains in cell culture (Table III).

The fourth group comprised 19 fecal specimens from patients with diarrhea and/or other symptoms. All were negative by virus culture and EM, and 18 (95%) were negative by PCR using both primers H1 and H2 or F1a and F2a. The four samples tested using primers JCH1 and JCH2 gave negative results. One sample was positive by PCR with primers F1a and F2a or H1 and H2, and revealed a slow growing adenovirus in culture using Graham 293 cells.

Overall, type 40 or 41 adenoviruses were detected in 76 fecal samples by IEM, REA, or subgenus F PCR, similar numbers (72 [95%] or 75 [99%]) being positive by EM or PCR, respectively. Nonetheless, perhaps more specimens with discrepant PCR and EM results were only PCR positive (n = 4) than vice versa (n = 1) ($P = 0.1$). Also, EM gave positive results for 13 other samples negative by F1a/F2a PCR and therefore most unlikely to contain subgenus F adenoviruses. Thus, EM was less specific and perhaps less sensitive than PCR for identification of the latter viruses. The PCR using primers F1a and F2a was more sensitive than IEM for detection of type 40 and 41 adenoviruses in specimens positive for these viruses by either technique (sensitivities 75/76 [99%] vs. 66/76 [87%], $P = 0.008$). The genus-reactive primer sets H1 and H2 or JCH1 and JCH2 were equally efficient for detection of adenovirus type 40 or 41 DNA in diarrheal stool specimens (sensitivities 70/75 [93%] vs. 14/15 [93%]). Stools positive by EM and subgenus F PCR but negative by IEM probably contained less adenovirus subgenus F particles than IEM positive specimens. The sensitivity of DNA amplification using genus-reactive primers was lower with the former rather than the latter samples (4/6 [67%] vs. 65/66 [98%] with primers H1 and H2, 2/3 [67%] vs. 11/11 [100%] with primers JCH1 and JCH2), though only with the first primer set was the difference statistically significant ($P = 0.03$ and $P > 0.1$, respectively). The sensitivities with the same 2 primer pairs were higher for IEM positive samples (65/66 [98%] or 11/11 [100%]) than for specimens negative by subgenus F PCR, irrespective of whether the latter were positive by EM (8/13 [62%], $P < 0.001$, or 1/4 [25%], $P < 0.001$) or virus culture (10/20 [50%], $P < 0.001$, or 1/11 [9.1%], $P < 0.001$). Primer set H1 and H2 gave more positive results than primers JCH1 and JCH2 with specimens not containing subgenus F adenovirus DNA but positive by EM or virus culture (18/33 [55%] vs. 2/15 [13%], $P = 0.01$).

For stools likely to contain fewer adenovirus particles than those positive by IEM (e.g., samples negative by IEM but positive by subgenus F PCR, EM alone, or just virus culture), the positivity rates were similar with primers H1 and H2 (6/9 [67%], 8/13 [62%], and 10/20 [50%]) but possibly decreased progressively with the second genus-reactive primer set (3/4 [75%], 1/4 [25%], and 1/11 [9.1%], $P = 0.6$ for comparison of the first and third sensitivities but $P > 0.1$ for other comparisons).

All 10 stool specimens positive for subgenus F adenovirus DNA by PCR but negative by IEM or EM or not examined by EM were collected from patients aged less than 2 years with diarrhea and vomiting.

Typing of Adenovirus Subgenus F Strains Using PCR

Digestion of the PCR product with *Hha* I after DNA amplification using primers F1a and F2a correctly typed the two control type 40 and two control type 41 adenovirus strains. This technique identified 23 fecal adenoviruses, shown to be subgenus F but not typed by IEM, as type 40 (n = 4) or type 41 (n = 19) (e.g., see Fig. 1).

DISCUSSION

Positive results were obtained with all three primer sets for virtually all subgenus F adenovirus positive fecal specimens despite lengthy storage and multiple thawing and refreezing of the specimens to allow evaluation of other adenovirus detection techniques [Wood and Bailey, 1987; Wood et al., 1989a,b] and further storage of the extracts prior to PCR testing using primers JCH1 and JCH2. The preservation of intact target DNA in these specimens and extracts presumably reflected the large amount of virus at collection, up to 10^{11} virions per gram of feces [Allard et al., 1992]. The mixture of positive and negative F1a/F2a PCR results for EM positive IEM negative stool samples probably indicated the presence or absence of subgenus F virions in the original feces rather than degradation of small quantities of the latter in some specimens to levels below the PCR detection limit. Degradation during storage of small quantities of virions and/or target DNA could, however, have explained the low sensitivity observed with primers H1 and H2 or JCH1 and JCH2 in subgenus F PCR negative

TABLE III. Results of Retrospective Study on Fecal Samples Positive for Adenoviruses in Cell Culture*

Virus isolation	EM	PCR using primers F1a and F2a	PCR using primers H1 and H2	PCR using primers JCH1 and JCH2	No. of samples
Ad 1	−	+[a]	+	+	1
	−	+[a]	NT	NT	1
	−	−	+	+	1
	−	−	−	−	1
	−	−	−	NT	1
Ad 2	NT	−	−	NT	1
	−	−	+	−	3
	−	−	−	−	2
	−	−	+	NT	2
	−	−	−	NT	1
	NT	−	−	NT	1
Ad 3	−	+	−	NT	1
	NT	−	−	NT	1
Ad 4	−	−	+	−	1
Ad 5	NT	−	−	NT	1
Ad 6	−	−	+	−	1
Ad (not typed)	−	−	+	−	2
Ad (not typed)	−	−	−	NT	1

*Ad 1–6, (not typed): adenovirus types 1–6 or untyped. − = negative; + = positive; NT = not tested.
[a]Identical result on two DNA extracts.

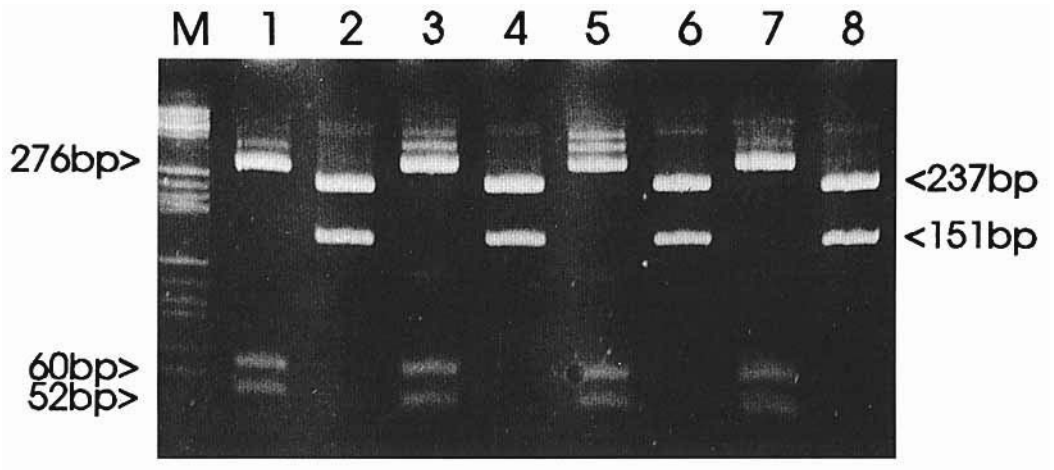

Fig. 1. DNA extracted from adenovirus IEM positive fecal specimens, amplified in the Fla/F2a PCR, and the product subjected to restriction endonuclease digestion using *Hha* I. **Lane M:** molecular weight markers (pBR322 *Hae* III digest); **lanes 1,3,5,7:** adenovirus type 41 DNA product; and **lanes 2,4,6,8:** adenovirus type 40 DNA product.

samples. The false negative results with these two primer sets for stool specimens containing type 2 or 5 strains were notable given the identity of the primer and target sequences [Kinloch et al., 1984; Allard et al., 1990; Hierholzer et al., 1993] and the high sensitivity of H1/H2 PCR with similarly positive conjunctival swabs [Morris et al., 1995]. The progressively lower sensitivity of the PCRs using primers JCH1 and JCH2 but not H1 and H2 with fecal specimens negative by IEM but positive by subgenus F PCR, EM alone, or just cell culture could have reflected destruction of the probably progressively smaller amounts of target DNA during storage of the extracts, rather than any difference in the true performance of the two PCRs. Too few experiments were carried out to define the frequency of DNA degradation in stored eye swab extracts noted herein, but a previous retrospective evaluation of H1/H2 PCR indicated that the problem was probably rare [Morris et al., 1995]. This problem might have explained the considerably lower sensitivity of JCH1/JCH2 PCR with the second of the two sets of conjunctival swab DNA extracts.

The higher efficiency of amplification of subgenus F adenovirus DNA using primers F1a and F2a rather than F1 and F2 illustrated the importance of minor changes of the primer sequences in increasing PCR sensitivity. A relatively low annealing temperature (40°C) failed to compensate for minor mismatching between the primer and target DNA sequences. Once the errors in the pub-

lished subgenus F-specific primers [Rousell et al., 1993] had been corrected, the resultant PCR proved to be at least as sensitive as EM for detection of type 40 and 41 adenovirus and more sensitive than IEM for subgenus classification of such strains. The selectivity of primers F1a and F2a was confirmed by the negative results with representative strains from all subgenera except F. The data confirmed the imprecision of assuming that all EM positive fecal samples contain subgenus F adenoviruses [Wood and Bailey, 1987] and the failure of monoclonal antibody-based IEM to type all subgenus F strains identified by polyclonal antibody-based IEM [Wood et al., 1989a], presumably because of greater avidity of the polyclonal antibodies. Both difficulties could be circumvented by using F1a and F2a primers to amplify adenovirus type 40 and 41 DNA in EM positive specimens and then digesting the resultant PCR products with *Hha* I to allow type identification. Adenovirus typing by REA of a subgenus F PCR product had not been described prior to our study, and would be valuable in plotting virus transmission in outbreaks [Richmond et al., 1979] and in documenting time-related differences in the prevalences of adenovirus type 40 or 41 infection [Bates et al., 1993]. The two genus-reactive DNA amplification assays were equally sensitive with subgenus F adenovirus DNA positive stool samples. Primers H1 and H2 or JCH1 and JCH2 were known to comprise sequences possessed by portions of the genes encoding the adenovirus type 40 and 41 hexon proteins [van Loon et al., 1987; Toogood et al., 1989]. Therefore, initial screening of stool specimens from patients with diarrhea or other symptoms using either of these genus-reactive primer pairs, followed by testing of the positive samples for subgenus F adenovirus DNA by F1a/F2a PCR and for other adenoviruses by cell culture, might be proposed. Unfortunately, both pan-reactive primer sets were perhaps less efficient than the subgenus F-specific primers for detection of type 40 and 41 adenovirus DNA and gave unacceptably low positivity rates with specimens containing other adenoviruses.

The PCRs using primers H1 and H2 or JCH1 and JCH2 amplified representative strains of all six adenovirus subgenera at annealing temperatures of 40°C [Turner et al., 1993; Morris et al., 1995] or 55°C [Hierholzer et al., 1993]. These PCRs achieved similar sensitivities (400 copies) with adenovirus type 2 genomic DNA as target [Morris et al., 1995]. Any differences between the two PCRs in assay performance with DNA extracts from clinical specimens tested concurrently probably reflected differential efficiencies of target DNA amplification with different adenovirus serotypes rather than any overall difference in amplification efficiency with the optimal target. With the first set of conjunctival swabs tested retrospectively, primer set JCH1 and JCH2 was more efficient than H1 and H2 with subgenus B and possibly non-subgenus B strains. The data accrued in the small prospective study of eye swabs were also consistent with JCH1 and JCH2 truly giving the more sensitive assay when H1/H2 PCR performed as well as in a previous study [Morris et al., 1995]. Nonetheless, the data from the second set of retrospectively tested swabs did not confirm this difference, perhaps because of target destruction during storage.

The PCR positive virus culture negative results on conjunctival swab or stool specimens probably reflected the presence of non-viable virus rather than contaminating adenovirus DNA in that the patients concerned had adenovirus-associated diseases. Furthermore, the F1a/F2a PCR was positive on fresh extracts of all stool specimens yielding subgenus F adenovirus DNA but negative by IEM or positive for a non-subgenus F adenovirus, and dual adenovirus subgenus F and non-subgenus F infections occurred occasionally [Wood and Bailey, 1987; Wood et al., 1988]. One sample positive by PCR using primers F1a and F2a but initially negative in standard virus culture and EM gave the results in further virus isolation studies expected with a subgenus F adenovirus strain [Wood and Bailey, 1987]. Thus, routine use of PCR-based diagnostic tests for adenoviruses appeared feasible provided meticulous techniques minimized the risk of specimen contamination with exogenous target DNA.

In conclusion, subgenus F adenovirus-specific PCR using primers F1a and F2a with *Hha* I digestion of the products could replace IEM and *Sma* I REA for subgenus identification and typing of type 40 and 41 adenoviruses in diarrheal stool specimens. Indeed, initial screening of rotavirus-negative samples using this PCR is an attractive alternative to EM, because the PCR is perhaps more sensitive and provides subgenus identification. Subgenus F adenovirus antigen detection by ELISA was not assessed in this study, but gave false negative results previously for IEM positive samples [Wood and Bailey, 1989b]. With conjunctival swabs, there was no evidence that initial PCR screening with primers H1 and H2 rather than primers JCH1 and JCH2 would increase the sensitivity. Alternative adenovirus primer sets will be required to improve the prospective detection of adenoviruses other than types 40 and 41 in clinical specimens.

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